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# Chromatographic and spectral studies of jetsam and archived ambergris

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Abstract

We describe determination of the dichloromethane-soluble components of 12 samples of the natural product, ambergris, using capillary gas chromatography-mass spectrometry (GC-MS). Ambergris is produced *in vivo* in about 1% of Sperm whales and is used in perfumery and for odour fixation. Whilst descriptions of ambergris chemistry appeared until about 40 years ago, few accounts of analyses of whole extracts of multiple samples of ambergris by GC-MS have been published before. As expected, our analyses revealed that the major component (up to 97% of the dichloromethane-soluble material) was ambrein, with co-occurring, variable proportions of steroids. Moreover, we report apparently for the first time, mass spectra and retention indices of derivatised ambrein. These data should now allow reliable, rapid confirmation of even small amounts of jetsam, archived museum and customs samples of ambergris and an assessment of ambergris 'quality'.

**Keywords:** Ambergris, Ambrein, Ambrox®, GC, GC-MS, Sperm whale.

## 1. Introduction

The coprolith, ambergris, which has been known for centuries, is produced in the rectum of 1% of Sperm and Pygmy Sperm whales (reviewed by Clarke 2006). On the death of the whale, ambergris is liberated into the sea and may stay in the ocean for prolonged periods before being washed ashore and beached, when it is termed 'jetsam ambergris'. Jetsam ambergris is valued by the perfumery industry as a fixative and odorant, but the properties ('quality') vary and this influences the prices attained for particular pieces. In the past, when commercial whaling was practised, large lumps of ambergris were also recovered from whale carcasses (Clarke 1954, 2006; Anderson et al. 2012 and references therein), although the fresh material reputedly has a disagreeable odour and rarely, or never, has the valued odour of the naturally weathered jetsam material. However, samples of jetsam ambergris are still found and sold (subject to the provisions of national laws in individual countries, which nonetheless sometimes still ban such trade (Anderson et al. 2012). Such jetsam samples may require verification by analysis. Museum samples of ambergris also require analysis at intervals; for example, to determine the effects of storage (Moniz and Hammond 1996) and customs determinations are sometimes required (Governo et al. 1977).

Even though a few studies of the volatile, odorous components of tinctures (ethanolic solutions) of aged ambergris have been made, usually after distillation (Mookherjee and Patel 1977; Awano et al. 2005) and it has long been known that the key component of fresh ambergris is ambrein ((I); Lederer et al. 1946), very few analyses of whole, unfractionated, lipid-soluble extracts of ambergris have ever been published, certainly by methods commonly in use today.

Ambrein (**I**) is probably produced by intestinal microorganisms in the whales (Clarke 2006), likely from the partial cyclisation of squalene (Oritani et al. 1970; Ueda et al 2013). Accounts of the proportions of ambrein (**I**) in individual ambergris samples vary, from a few percent, to most of the whole mass (Baynes-Cope 1962). Other organic constituents reported in early studies of whale ambergris (as opposed to jetsam) included pristane and metabolites of mammalian cholesterol metabolism, such as epicoprostanol (**III**); Lederer et al. 1946; Hardwick and Laws 1951; Baynes-Cope 1962). A variety of carboxylic acids, including a steroidal diacid, benzoic and stearic (octadecanoic) acids have been reported in saponified ambergris (Lederer et al. 1946; Hardwick and Laws 1951; Taha 1989). Inorganic constituents (in addition to fragments of squid beaks) identified after ashing, include up to 3% phosphate (Baynes-Cope 1962).

In summary, it is difficult from the existing, mostly rather old literature (Hardwick and Laws 1951), due to the lack of appropriate chromatographic and spectral data for whole extracts and the shortage of authentic ambergris samples with known provenance, to determine confidently, whether a jetsam sample is actually ambergris and if so, to assign the 'quality' or purity (Hardwick and Laws 1951). Isolation of ambrein (Hardwick and Laws 1951; Baynes-Cope 1962) and then assignment by nuclear magnetic resonance (NMR) spectroscopy (Moniz and Hammond 1996) is certainly possible, though qualitative, but details of the whole composition of extracts are then lost. Also, the amounts of material required for NMR spectroscopy can be quite large by comparison with combined chromatography-mass spectrometry methods, which is a particular disadvantage for analysis of valuable museum samples (Moniz and Hammond 1996; Lambert et al. 2000). In the present study we therefore examined two museum-archived samples of ambergris of known provenance, ancient perfumery samples and jetsam samples, by capillary gas chromatography-mass spectrometry (GC-MS) after derivatisation. In this way, we sought to provide information on the proportions and variations ('quality') of the major organic soluble constituents, such as ambrein (**I**) and epicoprostanol (**III**). Importantly, we also sought to record retention index and spectral data of derivatised ambrein, in order to provide a method for subsequent identifications.

## **2. Results and discussion**

Extraction and dissolution into dichloromethane, revealed that 25% to 47% of the whole masses of samples 1-3 collected from a Sperm whale in 1947 were dichloromethane-soluble (Table S1). The insoluble material was not examined further. Clearly there is considerable compositional variation, even within a single boulder of ambergris of known history. This is consistent with a previous study (Baynes-Cope 1962). For instance, the present data can be compared with those for ether-soluble portions, of what was probably the same boulder, which varied from 11-

96% (Laws, reported by Baynes-Cope 1962). Similarly, ether-soluble portions of an ambergris boulder collected in 1953 (Clarke 1954) varied from 80-98% (Baynes-Cope 1962); wide variations have also been reported for *ad hoc* pieces of unknown origin in the earlier literature. Many of the older analyses of the lipid-soluble fractions of ambergris relied on chromatographic isolation of ambrein and determination by melting point and gravimetry or rarely, infrared spectroscopy (Hardwick and Laws 1951; Baynes-Cope 1962). FTIR spectra of whole extracts of samples 1-3 obtained herein, were all broadly similar to each other (Figure S3) and exhibited the following features: a broad transmittance at 3372  $\text{cm}^{-1}$  was attributed to H-bonded hydroxyl O-H stretching. A weak transmittance at  $\sim 3067 \text{ cm}^{-1}$  was indicative of unsaturation and attributed to C-H stretch in an alkene. Transmittances at 2925 and 2863  $\text{cm}^{-1}$  were attributed to C-H stretching in methyl and methylene groups and those at 1461 and 1382  $\text{cm}^{-1}$  to the corresponding bending vibrations. Weak transmittances at 1711-1706  $\text{cm}^{-1}$  were indicative of ketonic carbonyl groups (C=O stretch). Transmittances at 1644  $\text{cm}^{-1}$  were attributed to C=C stretch and at 935 and 887  $\text{cm}^{-1}$  to C-H out of plane bends in alkenes. The spectra were similar in several aspects to that published by Governo et al. (1977) for a sample of ambrein (**I**) isolated from ambergris: the presence of the transmittance attributed to the keto group in the latter was not explained by those authors, but the other features are consistent with the known structure of ambrein and with the spectrum of synthetic ambrein (**I**). Similar spectra were also recorded herein for the archived perfumery samples 4-6 (Figure S3d-f) and suspected jetsam ambergris samples 7-11 (Figure S4a-e) and a further ambergris sample from a beached Sperm whale (sample 12).

Although previous attempts to form derivatives of ambrein proved problematic, in the late 1970s it was reported (Governo et al. 1977) that after chromatographic isolation from ambergris, ambrein could be converted to the trimethylsilyl (TMS) ether in the presence of 'Trisil Z' (trimethylsilylimidazole in pyridine, 1 h). However, those authors did not publish either the mass spectrum or the GC retention index of the derivatised products, thereby limiting the usefulness of the GC-MS method for general use, even for isolated ambrein. That study also preceded regular use of capillary GC (instead, less efficient Support Coated Open Tubular GC columns, were used).

High temperature GC herein showed no high molecular weight components were present in the dichloromethane-soluble fractions so we treated extracts of ambergris samples 1-3 with a Trisil equivalent *N,O*-bis(trimethylsilyl)acetamide + trimethylchlorosilane + *N*-trimethylsilylimidazole (3:2:3; 1 h) and examined the products by conventional capillary GC-MS (Figure 1). Each sample contained at most five components (A1-D); the extracts were relatively simple (Figure

1). As expected, the most distinctive component was ambrein (Figure 1, I; peak D), which had been converted efficiently to the TMS ether, as revealed, apparently for the first time herein, by the mass spectrum of the ether (Figure 1). The spectrum was very similar in many respects, to that of underivatised ambrein (Governo et al. 1977; Ueda et al. 2013) but notably and importantly, unlike that of ambrein, contained low abundance but diagnostic ions, including a molecular ion at  $m/z$  500 ( $M^+$ ) and an ion at  $m/z$  485 ( $M^+$ -methyl). A significant ion also appeared at  $m/z$  143, attributed to a mono-unsaturated,  $C_4H_6$ -OTMS moiety. The retention index on HP-5MS stationary phase was 3110; again this has not been reported previously to our knowledge and should aid future assignments.

Component C (Figure 1) had a mass spectrum and retention index comparable to those of epicoprostanol (III) TMS ether (available in a NIST mass spectral library) rather than coprostanol, which is somewhat unusual, but consistent with many earlier reports (Lederer et al. 1946; Baynes-Cope 1962). A further underivatised minor component in these three samples, was assigned to coprostanone (Figure 1, peak B, IV). The presence of the latter probably explained the observation of a transmittance assigned to a keto group in the infra red spectra (Figure S3). Probably, the presence of these steroids is due to the faecal nature of the origins of ambergris, since such compounds are well known metabolites of cholesterol metabolism and bacterial conversion of cholesterol metabolites. Interestingly, the proportions of steroids in samples 1-3 varied considerably (Figure 1). Whereas the steroids dominated sample 1 (Figure 1; Table S2), the extract of the inner core (sample 3) comprised almost pure ambrein (Figure 1; Table S2). Since this sample also had the highest proportion of dichloromethane-soluble material of samples 1-3, this ambergris sample comprised almost 50% ambrein by mass. Previous reports involving isolation and weighing of chromatographic fractions (Baynes-Cope 1962) showed ambrein contents of 14-40% in one boulder (collected 1953; ambrein/epicoprostanol ratios of 0.8 to 4) and 1-28% in another (ambrein/epicoprostanol ratios of 0.7 to 2.5). Two minor unknown isomers (Figure 1; peaks A1, A2) were also detected in each of samples 1-3 (Figures S6 and S7).

An extract of a small fragment (Sample 12) of ambergris obtained from a dead male Sperm whale beached near Texel, Netherlands, was examined in the same manner to the above analyses. The material was almost entirely soluble in dichloromethane (Table S1). GC-MS revealed essentially the same components A1-D, but the chromatogram was dominated by ambrein TMS ether (Figure 1), similar to sample 3. It is not known whether the fragment originated from the core of the boulder.

Dissolution of three archived perfumery samples of ambergris (samples 4-6) in dichloromethane, dissolved most of each sample (90-100%; Table S1). A similar treatment of the dichloromethane extracts with derivatising reagent, followed by GC-MS, produced chromatograms, which were similar to those of samples 1 and 2. GC-MS of the soluble portions indicated that ambrein-TMS ether was present as the single largest component (Figure 1, peak D). The steroids (Figure 1, peaks B, C, III, IV) were present (ambrein/epicoprostanol ratios >1 to 3). From the similarity of samples 4-6 with samples 1 and 2, the literature data and differences to jetsam samples 7-11, we conclude that these ancient samples probably derived from whale (rather than jetsam) ambergris.

We treated all five extracts of New Zealand jetsam samples 7-11 with derivatising reagent and examined the products by GC-MS (Figure 1). These each contained ambrein-TMS as the major constituent (ambrein/epicoprostanol about 6 to >30). The content of the steroids was very low (Figure 1) similar to whale samples 3 and 12 (Table S2). Such data may well be typical for jetsam ambergris; to our knowledge, no other similar data have been published.

### 3. Conclusion

As a result of the present analyses of 12 ambergris samples, we suggest that a reasonable protocol for verification of jetsam ambergris is the deployment of GC-MS, ideally with cold on-column injection, of organic extracts treated with a multiple silylation reagent (Governo et al 1977). Verification of the presence of ambrein-TMS ether as a major constituent, from the retention index and mass spectral data published herein would allow confirmation of an unknown jetsam sample as ambergris. The presence of epicoprostanol and cholestanone, identifiable by mass spectral comparison with widely available library and literature data for the TMS ethers, in addition to ambrein, might provide an estimate of the 'quality'; in the five jetsam samples analysed herein these were only minor components.

**Acknowledgements** We are grateful to Richard Sabin and colleagues of the Natural History Museum, London and Arthur Oosterbaan, curator of Ecomare Musuem, Texel, for access to whale ambergris samples and to Dr. Tony Curtis (University of Plymouth) and Drs. Beverly Bayne, Katie Aldridge and colleagues at CPL Aromas Ltd for supply of archived perfumery samples of ambergris and the latter for authentic samples of ambrinol and Ambrox®. We thank Professor Sato, Niigata University, Japan, for an authentic sample of ambrein and an anonymous supplier for the samples of New Zealand jetsam ambergris.

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## SUPPLEMENTARY MATERIAL

### Chromatographic and spectral studies of jetsam and archived ambergris

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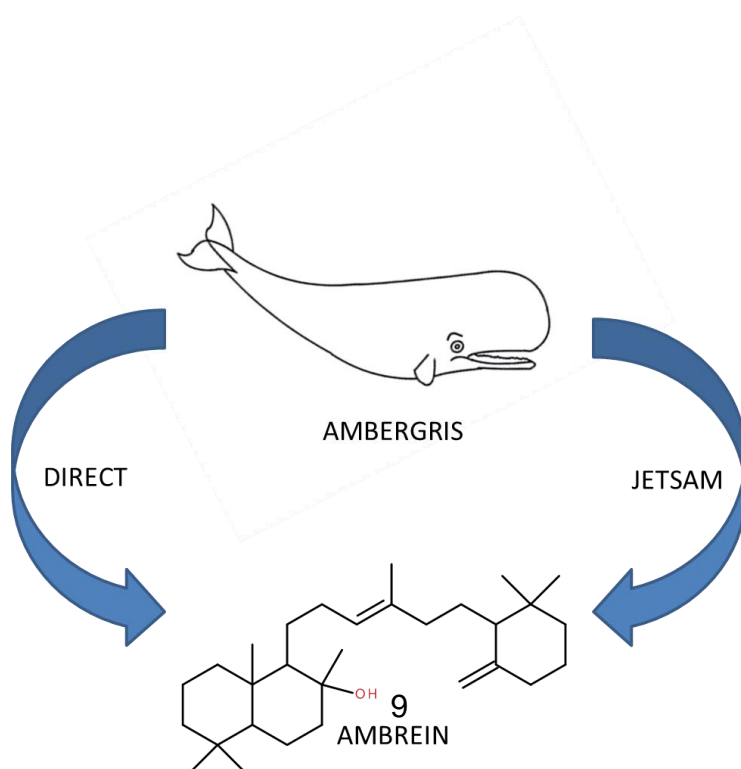
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266 Abstract

267 We describe determination of the dichloromethane-soluble components of 12 samples of the  
268 natural product, ambergris, using capillary gas chromatography-mass spectrometry (GC-MS).  
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271 few accounts of analyses of whole extracts of multiple samples of ambergris by GC-MS have  
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274 proportions of steroids. Moreover, we report apparently for the first time, mass spectra and  
275 retention indices of derivatised ambrein. These data should now allow reliable, rapid  
276 confirmation of even small amounts of jetsam, archived museum and customs samples of  
277 ambergris and an assessment of ambergris 'quality'.

278 **Keywords:** Ambergris, Ambrein, Ambrox<sup>®</sup>, GC, GC-MS, Sperm whale.

279

280 **Experimental**

281 **Materials**

282 Sample descriptions are shown in Table 1. A boulder of ambergris weighing 155 kg taken from  
283 a 16 m male Sperm whale on board Floating Factory ship *Southern Harvester* in 55°59'S,  
284 03°02'E on 21 November 1947 by Clarke [Clarke, 2006 shows a photograph taken at the time],  
285 portions of which are archived by the Natural History Museum, London, were sub-sampled by  
286 one of the present authors (SJR) on 14 June 2016. Small pieces were taken from an odorous  
287 black fragment with a smell similar to that of synthetic Ambrox<sup>®</sup> (II), covered in white crystals  
288 (Figure S1a; Sample 1); two further sub-samples were taken from the black outer laminae and  
289 the golden brown inner core of a further odorous piece (Figure S1b; Samples 2 and 3).  
290 Evidence of black shiny fragments, of what were assumed to be squid beaks, was visible in the  
291 outer part of the latter piece (Figure S1b). Samples were taken from the outer (Sample 2) and  
292 inner (Sample 3) parts of this piece (Figure S1b). These pieces are thus similar to those  
293 designated A-C by Laws, for which (non-GCMS) data (obtained by the method of Hardwick and  
294 Laws 1951) were reported by Baynes-Cope (1962).

295 Three, non-odorous, ancient archived samples of suspected ambergris were donated by  
296 sources from the perfumery industry (samples 4-6). Five samples of brown to white, slightly  
297 odorous suspected jetsam ambergris were provided from beaches from undisclosed locations in  
298 New Zealand (samples 7-11). An extract of a small fragment (sample 12) of over 83 kg of  
299 ambergris obtained from a dead male Sperm whale beached on 15 December 2012 at Razende  
300 Bol near Texel, Netherlands and archived by the Ecomare Museum, Texel was also provided by  
301 the museum. An authentic sample of ambrein was obtained from Ueda et al., (2013) and  
302 samples of ambrinol and ambroxan were supplied by a perfumery company. *N,O*-  
303 bis(trimethylsilyl)acetamide + trimethylchlorosilane BSTFA/TMCS (99:1) and *N,O*-  
304 bis(trimethylsilyl)acetamide+ trimethylchlorosilane + *N*-trimethylsilylimidazole; BSA +  
305 TMCS+TMSI (3:2:3) reagents were supplied by Sigma-Aldrich (Poole, UK).

306 **Methods**

307 Samples were taken from specimens of ambergris using an acetone rinsed scalpel blade and  
308 spatula and digested by sonication (2 x 5 min) in dichloromethane at a nominal concentration of  
309 10 mg mL<sup>-1</sup> prior to analysis using Fourier transform infrared spectroscopy (FTIR). Aliquots of

the digest were taken and diluted for direct analysis of the underivatized sample, or dried under a gentle stream of nitrogen (40 °C) and derivatized to their trimethylsilylated derivatives with BSTFA/1% TMCS (TMS; 50 µL; 70 °C; 1 h) or with BSA/TMCS/TMSI (3:2:3; 'TRISIL'; 100 µL; 70°C; 1 h) and reconstitution in solvent, prior to analysis.

FTIR spectroscopy was undertaken using a Bruker Alpha Platinum ATR (Bruker (UK) Ltd., Coventry, UK) instrument in transmittance mode (32 background and sample scans; resolution 4 cm<sup>-1</sup>) and data were collected from 4000 to 375 cm<sup>-1</sup>.

GC-MS was carried out using an Agilent GC-MSD (Agilent Technologies, Wilmington, DE, USA). This comprised a 7890A gas chromatograph fitted with a 7683B Series autosampler and a 5975A quadrupole mass selective detector operated at 70eV ionisation voltage. The column was a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.0 mL min<sup>-1</sup>. A 1.0 µL sample was injected into a 250 °C splitless injector. The oven temperature was programmed from 40 to 300° C at 10 °C min<sup>-1</sup> and held for 10 min.

HTGC-FID was carried out using an Agilent 6890 gas chromatograph fitted with cool-on-column inlet (0.5 µL manual injection; +3 °C track oven mode), high temperature FID jet (435 °C) and Agilent VF-5ht Ultimetall column (15 m x 0.25 mm i.d. x 0.1 µm; constant flow mode, helium carrier gas at 1 mL min<sup>-1</sup>). The oven was programmed from 40 - 430 °C at 10 °C min<sup>-1</sup> with 10 min hold. Samples were heated in a heater block (70 °C, 1 h) prior to hot injection.

High temperature (HT) GC-MS was carried out using a BenchTOF-dx™ reflectron time-of-flight mass spectrometer (Almsco International, Llantrisant, UK) interfaced with an Agilent 6890 gas chromatograph (set up in the same manner as HTGC-FID with an Agilent VF-5ht Ultimetall column (15 m x 0.25 mm i.d. x 0.1 µm; constant flow mode, helium carrier gas at 2.5 mL min<sup>-1</sup>), via an in-line Siltite™ mini-union and HT-deactivated silica tubing (2 m x 0.18 mm id ; Phenomenex, Macclesfield, UK). General operating conditions were: helium carrier gas; oven programmed from 40 - 430 °C at 20 °C min<sup>-1</sup>, 5 min hold; transfer line and ion source at 350 °C; mass spectrometer in EI mode (70 eV or 10 eV) recording mass range *m/z* 50 -1350. The chromatograph was controlled through Agilent MSD Chemstation (VE.02.01) and the spectrometer through ProtoTOF™(V 1.1.1) software. Data processing software included dx-Connect™ and TargetView™ with library matching via NIST/MS Search. Prior to operation, air/water background, signal optimisation and mass calibration (PFTBA) were performed using auto-routines (ProtoTOF software).

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Sample number	Origin	Date of collection (receipt)	% dichloromethane-soluble
1	Piece of ambergris from a boulder weighing 155 kg ex 16 m male Sperm whale Floating Factory ship Southern Harvester 55°59'S, 03°02'E 21 November by Clarke (2006). Black outer (Figure S1a).	1947	25
2	Piece of ambergris from a boulder weighing 155 kg ex 16m male Sperm whale Floating Factory ship Southern Harvester 55°59'S, 03°02'E 21 November by Clarke (2006). Black outer of inner core (Figure S1b).	1947	37
3	Piece of ambergris from a boulder weighing 155 kg ex 16m male Sperm whale Floating Factory ship Southern Harvester 55°59'S, 03°02'E 21 November by Clarke (2006). Golden brown inner core (Figure S1b).	1947	47

4	Archived perfumery sample (T Curtis).	(2016)	100
5	Archived perfumery sample (T Curtis).	(2016)	93
6	Archived perfumery sample (CPL).	(2016)	90
7	Jetsam sample New Zealand (White).	(2016)	93
8	Jetsam sample New Zealand (Sweet/woody).	(2016)	99
9	Jetsam sample New Zealand (Dark).	(2016)	103
10	Jetsam sample New Zealand (White-gold).	(2016)	99
11	Jetsam sample New Zealand (Silver)	(2016)	101
12	Small fragment of 83 kg of ambergris obtained from dead male Sperm whale beached on 15 December at Razende Bol near Texel, Netherlands, archived by Ecomare Museum, Texel.	2012 (2016)	93

**Table S1** Sample descriptions and % dichloromethane-soluble material.

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Sample	A1	A2	B	C	D
1	6	2	18	47	26
2	3	1	6	22	68
3	1	<1	3	8	87
4	3	2	6	25	64
5	3	2	5	46	45
6	2	1	5	37	55
7	<1	<1	<1	5	95
8	1	1	<1	3	95
9	1	1	4	8	85
10	2	1	1	12	85
11	<1	<1	<1	2	97
12	1	1	2	3	93

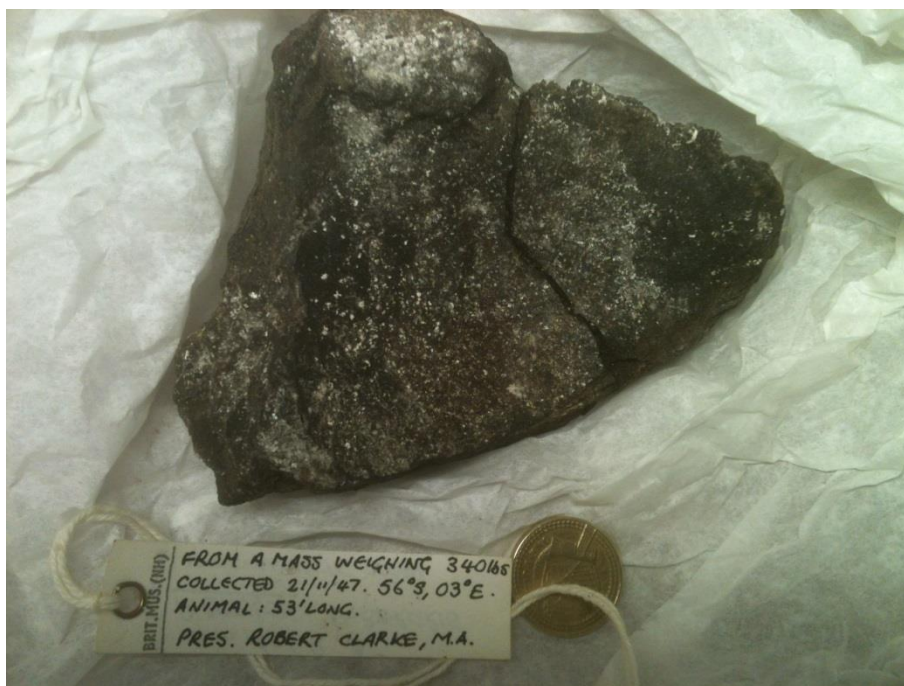
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349 **Table S2** Relative percentage compositions of components A1-D in dichloromethane soluble  
350 portions of ambergris samples 1-12. The proportions of dichloromethane extractable material in  
351 samples 1-12 are given in Table S1. A1,2=unknowns; B=coprostanone; C=epicoprostanol;  
352 D=ambrein.

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**Figure S1a.** Piece (sample 1) of ambergris from a boulder of ambergris weighing 155 kg taken from a 16 m male Sperm whale on board *Southern Harvester* in 55°59'S, 03°02'E on 21 November 1947 (Clarke 2006).



**Figure S1b.** Piece (samples 2 & 3) of ambergris from a boulder of ambergris weighing 155 kg taken from a 16 m male Sperm whale on board *Southern Harvester* in 55°59'S, 03°02'E on 21 November 1947 (Clarke 2006).









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382 **Figure S2** Five samples of white to silver jetsam ambergris collected from beaches from  
383 undisclosed beach locations in New Zealand (samples 7-11).

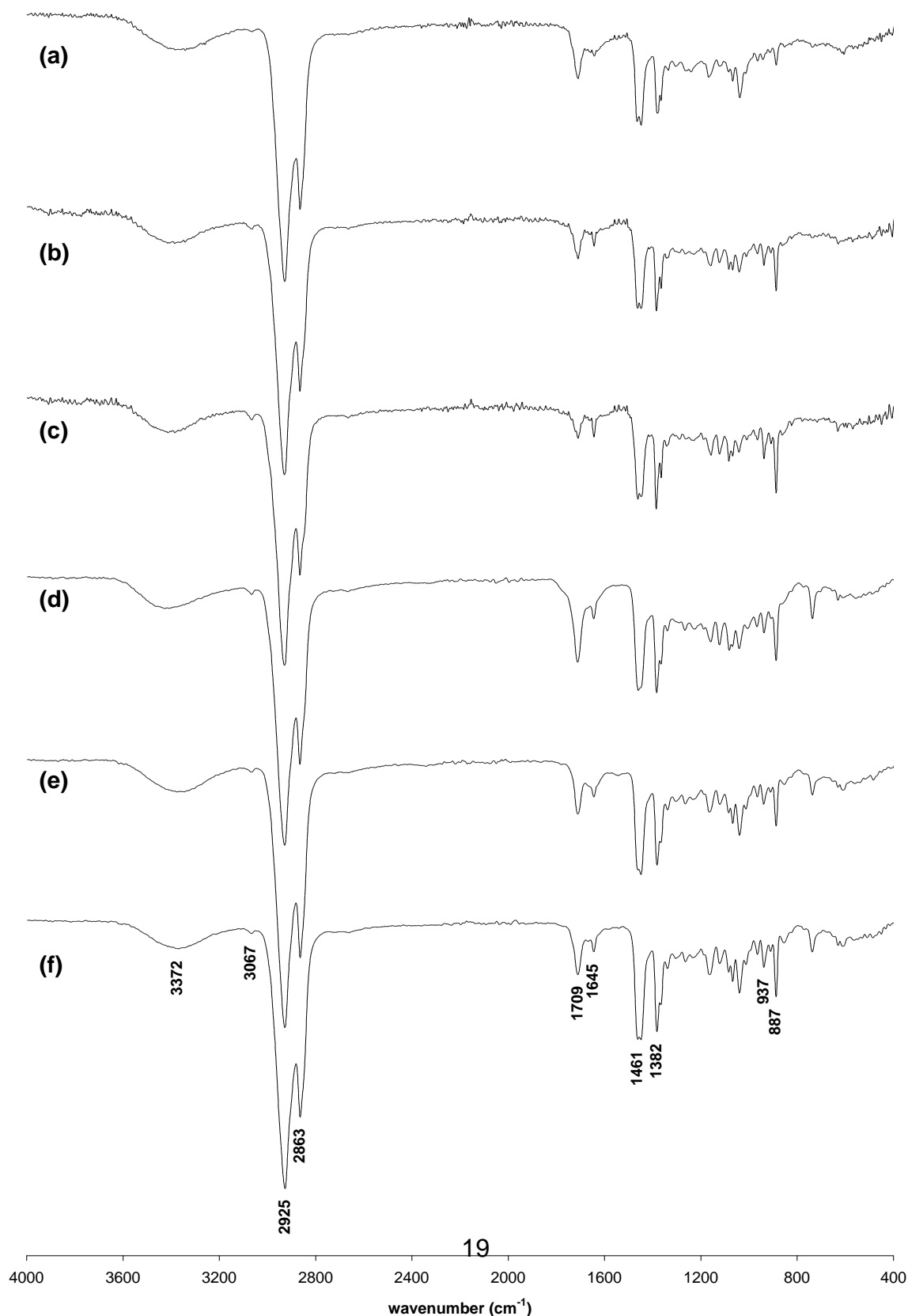
Sample 7 White		Hard, brown material with white surface coating
Sample 8 Sweet/woody		Hard, brown material
Sample 9 Dark		Hard, dark brown material with white surface mottle
Sample 10 White-gold		Hard, white exterior with gold/brown core

Sample 11 Silver		Hard, white
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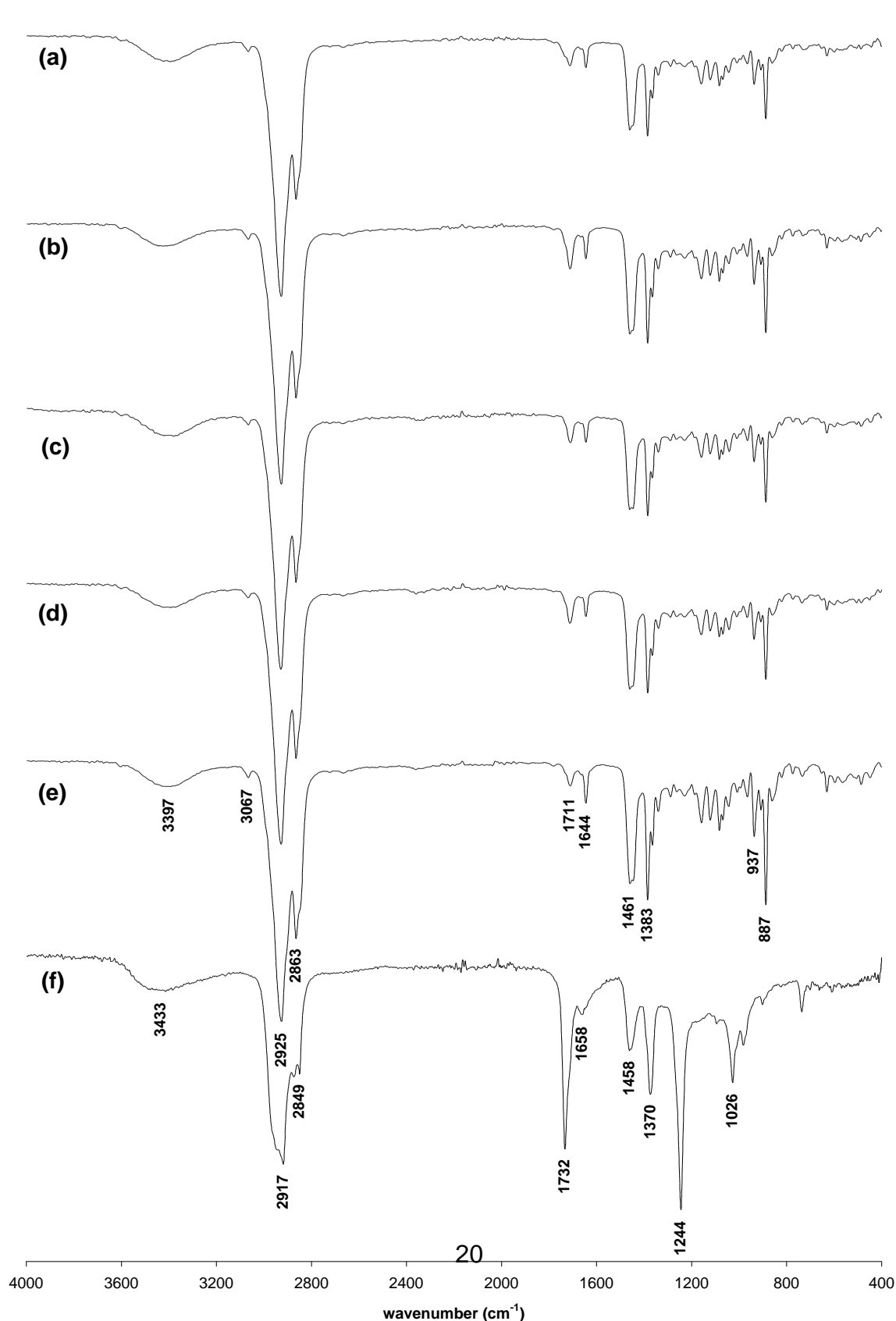
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**Figure S3.** FTIR spectra of (a-c) extracts of samples 1-3 from a boulder of ambergris weighing 155 kg taken from a 16 m male Sperm whale on board Floating Factory *Southern Harvester* in 55°59'S, 03°02'E on 21 November 1947 by Clarke (2006); (d-f) archived perfumery samples (Samples 4-6) of suspect ambergris.



**Figure S4.** FTIR spectra of (a-e) extracts of jetsam ambergris collected from beaches in New Zealand (samples 7-11); (f) sample of suspect jetsam ambergris bequeathed in Natural History Museum London in 1928 collected from Tanna Island, South Pacific (probably collected 1875). The latter is not ambergris.



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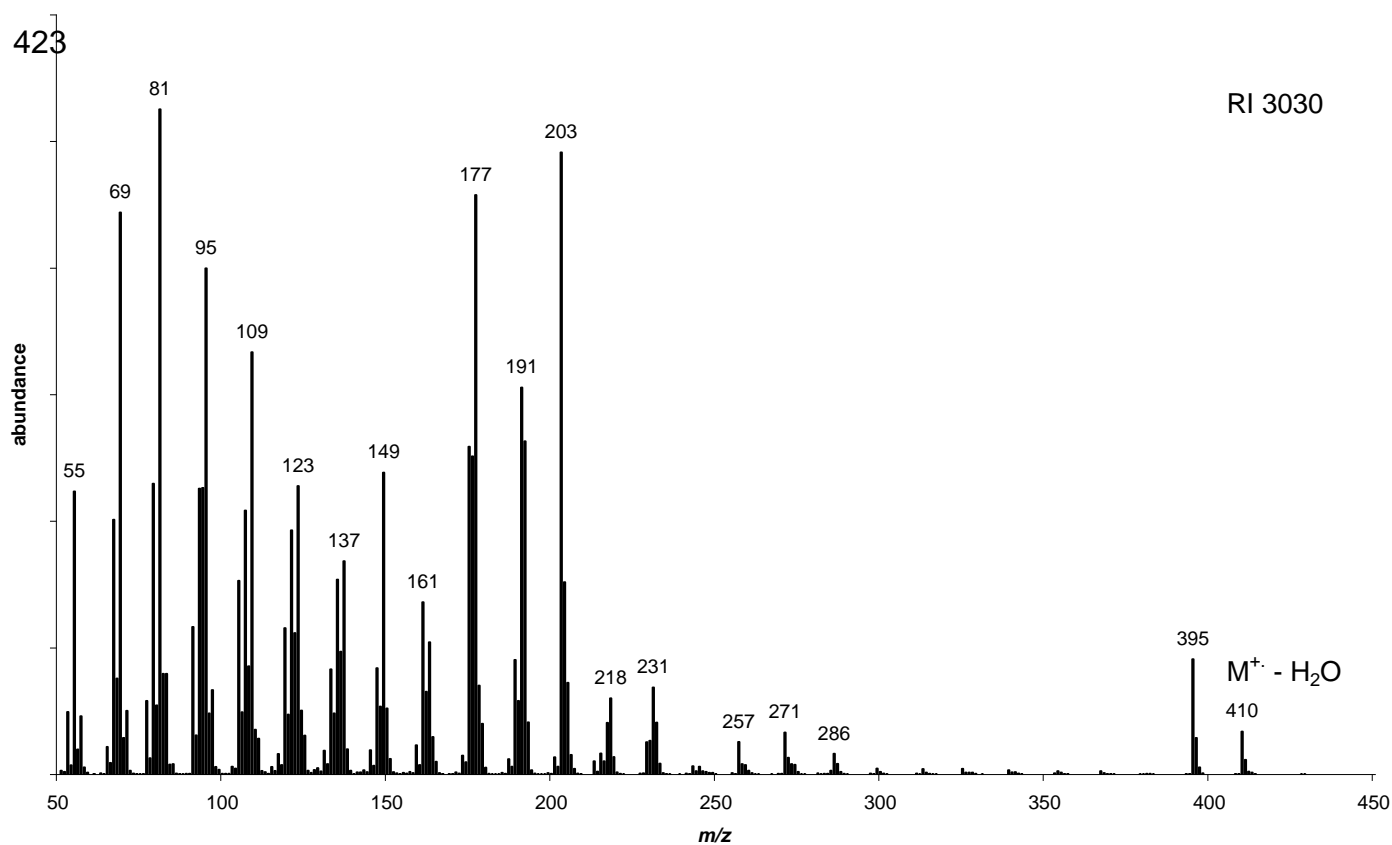
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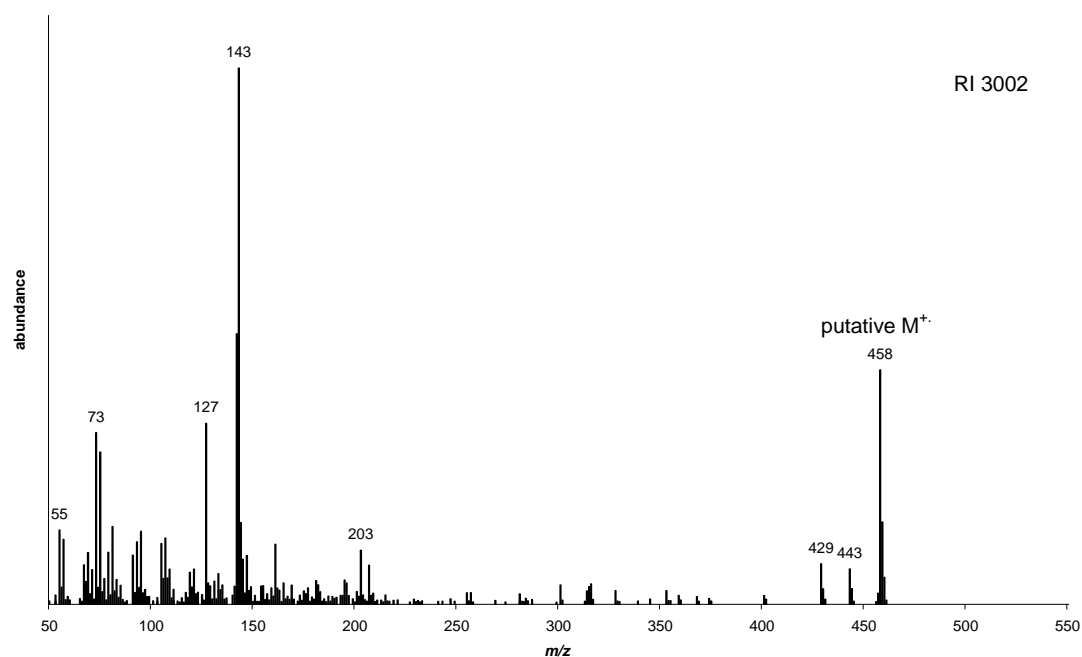
420 **Figure S5** Mass spectrum of underivatised ambrein (70eV, GC-MS). When derivatised this is  
421 component D.

422

423

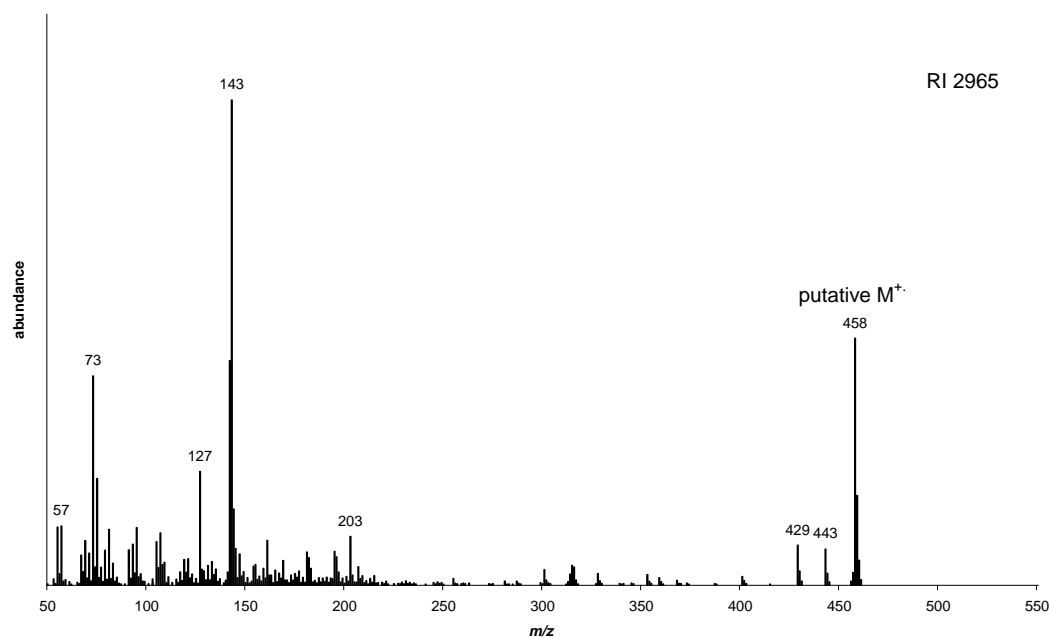


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425

426 **Figure S6.** Mass spectrum of unknown component A1 (as shown in Figure 1)



427

428 **Figure S7.** Mass spectrum of unknown component A2 (as shown in Figure 1)

429 We are grateful to Deniz Koseoglu (University of Plymouth) for supplying an original signed  
 430 copy of the previously reportedly partially mis-translated, Russian book, 'Cachalot' by A.A.  
 431 Berzin (1971) and for re-translating pages 320-324 on the composition of ambergris, into  
 432 English.

433

434 **Figure 1.** Structures (I-IV) of chemicals discussed in the text; total ion current GC-MS  
435 chromatograms of silylated ambergris dichloromethane whole extracts (samples 1-3  
436 and 12 from Sperm whales; samples 4-6 from archived perfumery sources and samples  
437 7-11 jetsam ambergris from New Zealand beaches). Components A1,2 are unknowns;  
438 component B is coprostanone; Component C is epicoprostanol TMS ether and  
439 Component D is ambrein TMS ether; electron impact mass spectrum of ambrein-  
440 trimethylsilyl ether.

441



